www.sciencemag.org/content/347/6224/842/suppl/DC1

Supplementary Materials for

Chemiexcitation of melanin derivatives induces DNA photoproducts long after UV exposure

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Published 20 February 2015, *Science* **347**, 842 (2015)
DOI: 10.1126/science.1256022

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Movies S1 and S2

Materials and Methods

Cells and Culture Media

Murine cells. Eumelanin-containing C57BL/6 melanocyte, melan-c albino melanocytes, and NIH3T3 fibroblasts were from the Specimen Resource Core, Yale SPORE in Skin Cancer. C57BL/6 melanocytes were cultured in OptiMEM (Cat. 31985-070, Life Technologies, Carlsbad, CA) supplemented with 7% horse serum (Cat. 100-508, Gemini Bio-Products, W. Sacramento, CA), 10 ng/ml (16.2 nM) TPA (12-O-tetradecanoylphorbol-13-acetate, Cat. P1585, Sigma, St. Louis, MO) and penicillin/streptomycin (100 U/ml penicillin and 100 µg/mL streptomycin, Cat. 15140-122, Life Technologies). Melan-c albino melanocytes were cultured in the same medium; in some experiments medium was supplemented with 5% FBS (Cat. F4135, Sigma), which was determined not to affect the induction of delayed CPD. NIH3T3 fibroblasts were cultured in DMEM High Glucose (Cat. 11965-092, Life Technologies) supplemented with 15% FBS, 100 U/ml penicillin, and 100 µg/mL streptomycin.

Human cells. Primary human melanocytes were isolated from fresh lightly-pigmented newborn foreskin tissue (*36*). Melanocytes were cultured in OptiMEM containing 100 U/ml penicillin, 100 µg/mL streptomycin, 5% fetal bovine serum (Cat. F4135, Sigma), 10 ng/ml TPA, 0.1 mM IBMX (Cat. 15879, Sigma), 0.1 mM dbcAMP (Cat. D0627, Sigma), 2.5 nM cholera toxin (Cat. C3012, Sigma), and 1 µM Na_3VO_4 (Cat. 450243, Sigma) and maintained in this medium for 1-2 weeks until a pure melanocyte culture is obtained. The melanocytes were then grown in OptiMEM supplemented with 100 U/ml penicillin, 100 µg/mL streptomycin, 5% FBS, 10 ng/ml bFGF (ConnStem Inc., Cheshire, CT), 1 ng/ml heparin (Cat. H3149, Sigma), 0.1 mM dbcAMP, and 0.1 mM IBMX with medium changes 3/week.

Mice

For *in vivo* experiments, UVA-irradiated mouse skin was the kind gift of Drs. Marcus Bosenberg and Viswanathan Muthusamy, Yale University School of Medicine. Experiments used the c-Kit ligand transgenic mouse (B6.Cg-Tg(KRT14-Kitl*)4XTG2Bjl/J) (Jackson Laboratory, Bar Harbor, ME), which expresses the ligand under control of the K14 promoter to limit expression to epithelial tissues. This mouse features melanocytes distributed throughout the basal layer of epidermis (*37*); in post-neonatal wildtype mice, melanin is present only in hair follicles. Some *K14-Kitl* mice were crossed with homozygous $Mc1r^{e/e}$ mutant mice having gold-colored fur (Jackson Laboratory). Dorsal skin of 4-5 week old mice was shaved and exposed to UVA (100 kJ/m^2) under isoflurane anaesthesia. Skin specimens were obtained at time 0 h and 2 h post exposure, fixed in formalin, and embedded in paraffin. All animal procedures were carried out in accordance with the Yale University Institutional Animal Care and Use Committee.

UV Sources and Dosimetry

The UVA (315-400 nm) source consisted of seven 20 watt F20T12BL lamps (Spectra Mini, Daavlin, Bryan, OH) passed through a 3 mm plexiglass plate filter and a BD Falcon polystyrene petri dish lid to remove UVC (100-280 nm) and most UVB (280-315 nm). As measured with an IL-1790 spectroradiometer (International Light, Peabody, MA), the incident radiation was 99.2 % UVA, 0.8% UVB, and 1×10^{-3} % UVC, with a UVA output of 60 J/m^2/s. The UV output was measured prior to each session using a UVX Radiometer (Cat. 97-0015-02, UV Products Inc., San Gabriel, CA) with UVA probe.

The narrowband UVB (nbUVB) source consisted of two 20 watt TL20W/01 Philips UVB Narrowband UVB lamps (Solarc Systems, Barrie, ON, Canada) having an output peak at 311 nm and nearly zero output outside the range 305-315 nm. The nbUVB source output was 2.9 J/m^2/s. The UV output was measured prior to each session using a 500C radiometer (National Biological Corp., Twinsburg, OH).

Cells at 70-80% confluency were exposed to UVA in cold PBS on ice (27.75 min for the 100 kJ/m^2 exposure), with the culture dish lid in place. For consistent results, it is essential to maintain reproducible confluency, oxygen levels (oxygen is a triplet quencher), and cooling. For the shorter nbUVB exposures (11.5 min for 2 kJ/m^2), cells were exposed in room temperature PBS with the lids off to avoid UVB absorption. After exposure, cells were returned to the incubator in complete medium at 37 ^{0}C and harvested at various times. For both UV wavelength regions, most experiments used a dose giving 75-85% cell survival as determined by colony assay.

ELISA for Cyclobutane Pyrimidine Dimers (CPD)

ELISA for CPD was performed using mouse monoclonal anti-CPD (Cat. CAC-NM-DND-001, Clone TDM-2, CosmoBio, Inc., Carlsbad, CA), following the manufacturer's protocol (Cat. NMDND001). 96-well polyvinylchloride flat-bottom microtiter plates (Cat. 2801, Thermo Scientific, Waltham, MA) were coated overnight at 37^{0}C with 50 µl 0.05% protamine sulfate (Cat. P3369, Sigma) in distilled water. The dried plates were washed 3x with distilled water, dried at 37^{0}C, and stored in the dark at room temperature. DNA was isolated from cells using the DNeasy blood and tissue kit (Cat. 69581, Qiagen, Valencia, CA) and heat denatured using a PCR machine (PTC-100, MJ Research, St. Bruno, Quebec, Canada) set at a single cycle of 100^{0}C for 10 min, followed by snap cooling in ice water for 15 min. For each sample, triplicate wells received 50 µl of 1 ng/µl heat denatured DNA per coated well and were allowed to dry overnight at 37^{0}C. DNA-coated plates were washed 5 times with 150 µl PBST (0.05% Tween-20, Cat. P7949, Sigma, in 1x PBS) and blocked in 2% FBS in PBS (150 µl) at 37^{0}C for 30 min. CPD were detected by adding anti-CPD, diluted 1:1,000 in 2% FBS (100 µl per well), and incubating for 30 min at 37^{0}C. After 5 washes with PBST, the plate was incubated with 100 µl of a 1:2,000 dilution of Biotin-F(ab')2 fragment of anti-mouse IgG(H+L) (Cat. B-2763, Life Technologies) in 2% FBS for 30 min at 37 ^{0}C. Following 5 washes with PBST, the plate was incubated with Streptavidin HRP (Cat. 434323, Life Technologies; 100 µl of a 1:10,000 dilution in 2% FBS) for 30 min at 37^{0}C. The plate was washed 5x with PBST and once with citrate phosphate buffer (24.3 mM citric acid monohydrate, J.T. Baker Cat. 0110-01 and 51.4 mM sodium phosphate dibasic, Sigma Cat. S7907, pH 5.0). Horseradish peroxidase was visualized with the substrate *o*-phenylene diamine (Cat. P23938, Sigma; 400 µg/ml in citrate phosphate buffer, 100 µl per well) for 30 min at 37^{0}C. The colorimetric reaction was stopped with 2M H_2SO_4 and absorbance at 490 nm was determined using a Synergy 2 multimode microplate reader (BioTek, Winooski, VT).

Comet Assay for Cyclobutane Pyrimidine Dimers

The comet assay used the UVDE FLARE assay kit from Trevigen (Cat. 4100-100-FM, Trevigen, Gaithersburg, MD). UV-exposed and un-exposed control cells were trypsinized, washed with PBS, and re-suspended in cold PBS on ice. Cells were mixed at

5 x 10^5 cells/ml with low melting agarose (Trevigen Cat. 4250-050-02) and 45-50 μl of mix was plated onto Trevigen FLARE slides (Cat. 3950-075-02). Agarose was allowed to polymerize for ~10 min at 4 ^{0}C after which the slides were incubated overnight in pre-chilled lysis solution (Cat. 4250-050-01, Trevigen). Slides were rinsed three times for 10 min each with 1X Flare buffer 2 and incubated with a 1:10 dilution of *Schizosaccharomyces pombe* UVDE enzyme for 30-40 min at 37^{0}C to incise CPD (as well as (6–4) photoproducts, apurinic/apyrimidinic sites, uracil, dihydrouracil, and possibly base mismatches and insertion-deletion loops). After the repair enzyme treatment, slides were incubated in the dark in alkali solution pH>13 (300 mM NaOH, 1 mM EDTA) for 30 min with one change of the solution. Electrophoresis was performed in cold alkali solution (300 mM NaOH, 1mM EDTA) at 4^{0}C for 30 min at constant 20 V using the Trevigen electrophoresis system (Cat. 4250-050-ES). Slides were then washed with distilled water two times for 10 min each and dehydrated in 70% ethanol for 5 min. Finally the slides were dried at 45^{0}C for 15-20 min until the agarose was completely flat. The DNA comets were stained with 1x SYBR green (Cat. S7563, Life Technologies) and photographed with a digital inverted microscope (EVOS, Advanced Microscopy Group, Fisher Scientific). Comet analysis used CometScore Pro software from TriTek Corp. (Sumerduck, VA). All comet assays were repeated at least 2 times for each sample type.

siRNA Knockdown of Excision Repair

The *Xpa* and *XPA* genes are required for both global and transcription-coupled nucleotide excision repair; the *Xpc* and *XPC* genes are required for transcription-coupled nucleotide excision repair. All transfections were done in basal OptiMEM, free of serum and antibiotics, using Promofectin siRNA transfection reagent (Cat. PK-CT-2000-RNA-200, Promokine, Sacramento, CA). All siRNAs were from Santa Cruz Biotechnology (murine *Xpa:* sc-36854, *Xpc:* sc-37806; human *XPA:* sc-36853*, XPC:* sc-37805; control siRNA: sc-37007) and are pools of three to five target-specific 19-25 nt siRNAs. si*XPA* was transfected into donor C59 and si*XPC* into a pool of donors C176 and C177. On the day before transfection, cells were plated in 60 mm dishes so that they were ~60-70% confluent on the day of transfection. Cells were washed once with OptiMEM and 1 ml of basal OptiMEM was added. The siRNA complexes were made according to the Promokine protocol, using 300 nM of siRNA nucleotides for each target gene, incubated at room temperature for 15 min and added to the cells drop-wise. Cells were incubated with the siRNA complexes in basal OptiMEM in the cell culture incubator for 4-6 h, after which 1 ml of 2X complete cell culture medium was added. Medium was changed with 1x complete medium after 12 h. Cells were incubated for an additional 36 hours in the incubator, after which the mRNA knockdown level was assessed by RT-qPCR.

For RT-qPCR, total RNA was isolated from human melanocytes that had been incubated with target siRNA, control siRNA, or no siRNA, using the RNeasy kit (Cat. 74104, Qiagen). RNA was converted to first strand cDNA using the High Capacity Reverse Transcription Kit (Cat. 4368814) from Applied Biosystems, Carlsbad CA. qPCR primers were designed using NCBI's Primer-Blast (*www.ncbi.nlm.nih.gov/tools/primer-blast/*). The primer sequences were: Mouse *Xpa* Forward, GCAGCTCCCAAAATGATTGACACC; *Xpa* Reverse, TCCATGACGGGCCCTGGTTC; *Xpc* Forward, CAGGCGAAAGAACGGGAAAGA; *Xpc* Reverse, TGAACCTTGTGCATGTTCTCCTGTA. Human *XPA* Forward, CAACCAGGACCTGTTATGGAA; *XPA* Reverse, TGCAGTTATCACAAGTTGGCA;

XPC Forward, AACCTGCCCAATCTACACCG; *XPC* Reverse, CATCAGTCACGGGATGGGAG.

Immunohistochemistry for CPD in Skin Sections

Mouse skin specimens were fixed in formalin, then dehydrated, paraffin embedded, cut into 5 micron sections, and mounted on coated slides. Skin sections were baked at 60 ^{0}C for 1-2 h followed by deparaffinization in 3 changes of xylenes, 5 min each. Sections were rehydrated through 100%, 90%, and 70% ethanol with two 5 min washes in each, followed by two 2 min washes with water and one 5 min wash with PBS. For antigen unmasking, sections were placed in a Coplin jar of boiling 10 mM citrate buffer, pH 6.0 (Cat. H3300, Vector Laboratories, Burlingame, CA) and microwaved at 5 minute intervals to re-establish boiling over 15 min. After cooling down, the sections were washed for 10 min in PBS. DNA denaturation was done by dipping the sections in 0.2 mM NaOH in 70% ethanol for 30 min at room temperature followed by three 5 min PBS washes. Because the primary anti-CPD antibody was a mouse monoclonal, non-specific antibody binding was prevented using the "Mouse on Mouse" blocking kit (Cat. FMK-2201, Vector Laboratories). Blocking was done for 1 h followed by a 5 min wash with M.O.M diluent. All antibodies were diluted in M.O.M diluent as well. Skin sections were incubated with a 1:500 dilution of anti-CPD antibody (Cat. CAC-NM-DND-001, Clone TDM-2, CosmoBio) for 30-60 min at room temperature followed by three 5 min washes with PBS. This was followed by 30 min incubation with a 1:1000 dilution of biotin-tagged anti-mouse IgG (H+L, Cat. B-2763, Life Technologies) in M.O.M. diluent at room temperature. The signal was developed using the ABC system from Vector Laboratories (Cat. PK-4000). Counterstaining was performed with hematoxylin. Slides were then dehydrated through an alcohol series 70%, 90%, and 100%, and immersed in xylene for 5 min. After air drying, sections were mounted on slides using Permount (Cat. SP15-500, Fisher Scientific). For quantification, signals from hematoxylin and DAB (for CPD) were separated using the color deconvolution plugin of Image J. To ensure that the darkest nuclei were not saturated in intensity, DAB intensity was quantified as the percentage of pixels darker than threshold, with the threshold set so that the darkest positive nuclei in the sample collection contained some subthreshold pixels. Epidermal segments were demarcated and scored using Image J.

Quantification of DNA Photoproducts by HPLC–MS/MS

The DNEasy blood and tissue kit (Qiagen) was used to maximize melanin removal, following the manufacturer's instructions. Cells were lysed and the resulting suspension was loaded onto a DNA-binding column. After washing steps to eliminate RNA and protein, DNA was eluted in water. DNA was then digested with 0.025 units of phosphodiesterase II/2.5 units of DNase II/0.5 units of nuclease P1 at pH 6 for 2 h at 37°C. A second digestion step was performed using 0.05 units of phosphodiesterase I and 2 units of alkaline phosphatase at pH 8 for 2 h at 37°C. All enzymes were from Sigma (St. Quentin Falalvier, France). Next, 0.1 M HCl was added and the sample was centrifuged and transferred to HPLC vials. This solution contained normal bases as nucleosides, whereas the bipyrimidine photoproducts consisted of modified dinucleoside monophosphates. Cytosine-containing cyclobutane dimers were obtained as their uracil derivatives due to fast and quantitative spontaneous deamination.

Hydrolyzed DNA samples were freeze-dried overnight and the resulting residues were solubilized in 40 µl of 20 mM triethylammonium acetate (Sigma) just before analysis on HPLC-MS/MS. Samples were injected onto a series 1100 microHPLC system (Agilent Technologies, Massy, France) coupled to an API 3000 triple quadrupole mass spectrometer (PerkinElmer/SCIEX, Thornhill, ON, Canada). Separation was performed on an Uptisphere ODB octadecylsilyl silica gel column (150 x 2 mm; 5 μm particle size) from Interchim (Monluçon, France) with a gradient of acetonitrile in 2 mM triethylammonium acetate. Chromatographic conditions and mass spectrometry features were as described (*38*). Normal nucleosides were quantified by HPLC with the UV detector set at 270 nm. MS/MS detection relied on the monitoring of specific fragmentation reactions of the analytes in the triple quadrupole system. The signals used for detecting the bipyrimidine photoproducts, corresponding to conversion of the pseudo-molecular ion into a specific daughter ion, were: *m/z* 545 → 447 for the TT cyclobutane dimer, *m/z* 545→ 432 for the TT (6-4) photoproduct, *m/z* 531 → 195 for the TC and the CT cyclobutane dimers, *m/z* 530 → 195 for the TC (6-4) photoproduct, and *m/z* 517 → 195 for the CC cyclobutane dimer.

<u>Photochemical Pathways – Inhibitors and Probes</u>

Inhibitors: C57BL/6 melanocytes were incubated with 50 μM of tyrosinase inhibitor kojic acid (2-hydroxymethyl-5-hydroxy-γ-pyrone, Cat. K3125, Sigma) or various concentrations of NADPH oxidase inhibitor III (VAS2870, Cat. 492000, Millipore, Billerica, MA; IC_{50} = 10.6 μM against NOX activity in human neutrophil lysates; complete inhibition at 5 μM in HUVEC cells and 10 μM in PDGF-induced primary rat vascular smooth muscle cells) or iNOS inhibitor aminoguanidine hydrochloride (Cat. 81530, Cayman Chemicals; IC_{50} for mouse iNOS and rat nNOS = 5.4 and 160 μM) in complete cell culture medium for 24 h prior to UVA exposure and these agents were added back to the post-exposure medium.

Antioxidants: Cells were incubated with 10 mM of *N*-acetylcysteine (Cat. A9165, Sigma), 10 μM of TEMPOL (Cat. 176141, Sigma), or various concentrations of α-tocopherol (vitamin E, Cat. T3251, Sigma) in complete cell culture medium for 16-24 h prior to UVA exposure and these agents were added back to the post-exposure medium.

Quenchers: The triplet-specific quencher, sorbate ester (ethyl sorbate, Cat. 177687, Sigma) or the triplet energy acceptor 9,10-dibromoanthracene-2-sulfonate (DBAS, Cat. D3482, US Biological, Salem, MA, dissolved in DMSO at 20 mM) (*26*) were added to the cells in complete cell culture medium immediately after UVA exposure to give 10 ng/ml and 20 μM, respectively. Cells were then incubated for various times before collecting cell pellets for DNA isolation. Consequently there is no 0 h timepoint. This timing is necessary because ethyl sorbate resulted in 2-4 fold higher CPD formation at time 0 if pre-incubated with cells before UVA exposure, presumably due to collisional energy transfer. The same behavior was seen with α-tocopherol at concentrations >1 μM. DBAS was not present during UV exposure to avoid the same phenomenon.

<u>Immunofluorescence for CPD and Nitrotyrosine</u>

Primary antibodies: Mouse monoclonal anti-CPD antibody (Cat. CAC-NM-DND-001, Clone TDM-2, CosmoBio), used at 1:2,000 dilution; rabbit polyclonal anti-3-nitrotyrosine (Cat. A-21285, Invitrogen), used at 1:2,000 dilution.

Secondary antibodies: DyLight-488 Horse anti-Mouse IgG (Cat. DI-2488, Vector Laboratories); Alexa Fluor 488 Goat anti-rabbit IgG (Cat. A-11008, Life Technologies); and Alexa Fluor 488 Goat anti-Mouse IgM (Cat. A-21042, Life Technologies). All secondary antibodies were used at 1:1000 dilution.

After UVA exposures, cells were fixed with 4% buffered formalin (Cat. HT501128, Sigma) for 10 min, washed 3x in PBS for 5 min each, and then permeabilised with 0.2% Triton-X (AB02025-00500, American Bioanalytical) for 10 min, all at room temperature. Blocking buffer for non-specific antibody binding was a mixture of 5% goat serum (Cat. G9023, Sigma) and 2% IgG free BSA (Cat. 001-000-161, Jackson ImmunoResearch, West Grove, PA) in 0.1% Triton-X, incubated with fixed cells for 2 h at room temperature, followed by one PBS wash for 5 min. All antibodies were diluted in the corresponding blocking buffer. Primary antibodies were incubated overnight at 4 ^{0}C and fluorescence conjugated secondary antibodies were incubated for 1 h at room temperature with mild shaking. Vectashield (Cat. H-1000, Vector Labs) was used to prevent fluorescence quenching during imaging and microscopy was performed on a LSM510 Meta laser scanning confocal microscope (Carl Zeiss, Thornwood, NY). Fluorescence quantitation of CPD and nitrotyrosine was performed on 50-60 individual cells using Image J software.

<u>Estimation of UVA-induced Spike in Peroxynitrite Flux</u>

Protein half-lives average two days. Any persistent protein-modification signal is biased toward the longer-lived proteins, so we conservatively assume a 4-day average half-life. For dividing cells in culture, a nitration signal would last ~2 halflives before being turned over or diluted out by cell proliferation, so the basal nitration signal represents ~8 days of accumulation. UVA exposure extended for 27 min; its contribution was equal to the 8 day basal accumulation, so the flux per hour after UVA was 400 fold larger than basal.

<u>Ultraweak Chemiluminescence from UVA Exposed Cells</u>

The ultra-weak chemiluminescence from permeabilized cells was recorded using the Single Photon Counting protocol on a Tri-Carb Liquid Scintillation Analyzer with Quanta Smart analysis software (PerkinElmer). After UVA exposure, approximately $2x10^6$ cells were washed with PBS and collected by trypsinizing or scraping in 500 µl of 20 mM Tris-HCl pH 7.4 (Cat. AB14044-01000, American Bioanalytical) containing 200 µM DBAS and 2 mM EDTA. Immediately before photon counting, an equal volume of Tris-HCl containing 0.5% Tween was added. Reproducible mixing is important for consistent results because O_2 is a triplet quencher. Cell permeabilization continues for several minutes, so permeabilization and chemiluminescence recording initially proceed concurrently. Chemiluminescence was recorded over 10 min, with a reading every 25 s.

<u>Chemiluminescence from Lipid Oxidation</u>

Approximately $2x10^6$ C57BL/6 melanocytes were trypsinized and washed with ice cold PBS. To release nuclei, cells were incubated in cold hypotonic solution for 2-3 cycles of 5 min incubation followed by centrifugation and fresh hypotonic solution. The hypotonic solution was 10 mM HEPES (Cat. AB06021-00100, American Bioanalytical) pH 7.4-7.9, 10 mM KCl (Cat. AB01653-01000, American Bioanalytical), 5 mM $MgCl_2$ (Cat. AB09006-00100, American Bioanalytical), 0.5% NP-40 (Cat. I8896, Sigma) and, to prevent oxidative DNA damage, 5 mM deferoxamine methanesulfonate (Cat. D9533, Sigma) and 1 mM reduced L-glutathione (Cat. G4251, Sigma). Nuclei were harvested when approximately 90% nuclei yield was reached, as assessed by trypan blue staining. The isolated nuclei were washed twice with cold 10 mM HEPES, 250 mM sucrose (Cat S0389, Sigma) and twice with cold PBS, then treated with cumene hydroperoxide (Cat. 247502, Sigma) in PBS for 1 hr at room temperature. UVA exposure, DNA isolation, and ELISA were performed as described above.

Chemiluminescence from Melanin Oxidation

Synthetic tyrosine-derived melanin (0.1 to 0.5 mg/ml, Cat. M8631, Sigma) was oxidized using various concentrations of peroxynitrite (stabilized in 1.175 mM NaOH, Cat. 516620, EMD Millipore, Billerica, MA) in 50 mM phosphate buffer, pH 7.4 - 8.8 (or in water) or oxidized using 5 µM horseradish peroxidase (P8375 Sigma) plus 50 µM H_2O_2 (Cat. 2186-01, J.T Baker) in 50 mM phosphate buffer, pH 7.4 - 8.8; all reactions contained 50 µM DBAS. The chemiluminescence was recorded using the Single Photon Counting protocol over 10 min, with one reading every 25 s.

Eumelanin monomer 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and pheomelanin precursor 5-S-cysteinyldopa (5SCD) were synthesized as described (*39*). The DHICA analog 5-hydroxyindole-2-carboxylic acid (5OH2CA) was purchased from Sigma. These compounds were oxidized using the same protocol as for melanin.

Synthetic melanin, DHICA, 5SCD, and 5OH2CA were sparingly soluble in water or phosphate buffer at these pHs. Their suspension converted, upon oxidation with ONOO or HRP/H_2O_2, to a solution that was dark (melanin), purple-brown (DHICA), or light yellow (5SCD and 5OH2CA).

CPD Generation in Plasmid DNA or Oligonucleotides after Melanin Oxidation

Synthetic melanin (0.1 - 0.5 mg/ml) was oxidized using 300 µM peroxynitrite in 50 mM phosphate buffer pH 8.8 at room temperature or using 20 µM horseradish peroxidase with 200 µM H_2O_2 in 50 mM phosphate buffer pH 8.8 containing 2 mM EDTA The peroxynitrite reaction required pH 8.8 and the EDTA. To the melanin reaction was added 3-5 µg of pUC19 plasmid. The melanin oxidation was allowed to occur for 0.5-1 h with all reactants supplemented every 20 min so that the total concentrations were as stated above. Plasmid DNA was purified by passing it through OneStep™ PCR Inhibitor Removal Kit (Cat. D6030, Zymo Research, Irvine, CA). Purified DNA was used for CPD detection by ELISA. Similar reactions were carried out using the eumelanin monomer DHICA, the pheomelanin monomer 5SCD, and the DHICA analog 5OH2CA, all at final concentrations of 0.5-1 mg/ml. Reactions were carried out in the dark. Similarly, 5 µg of various single-stranded, heat-denatured oligonucleotides were added to the horseradish peroxidase / H_2O_2 oxidation reaction described above.

Oligonucleotides used for CPD measurement in melanin and melanin-monomer oxidations

Sites of expected CPD are underlined; oligo C-88 contains repeats of the melanoma mutation hotspot motif *(9)*.

Oligo Name	Sequence (5'-3')	Length (nt)	Types and number of CPD expected	Total CPD expected
N	CGCACGTATGCACGTACGCACGTATG CACGTACGCACGTATGCACGTACGCA CGTATGCACGTACGCACGTATGCACG TACGCACGTATGCACGTACGCACGTA TGCACGTACGCACGTATGCACGTACG CACGTATGCACGTACGCACGTATGCA CGTA	160	TT = 0 TC = 0 CT = 0 CC = 0	0
C- 21	GTCTCGCGCGCGCGACGCGGTCTCGC GCGCGCGACGCGGTCTCGCGCGCGCG ACGCGGTCTCGCGCGCGCGACGCGGT CTCGCGCGCGCGACGCGGTCTCGCGC GCGCGACGCGGTCTCGCGCGCGCGAC GCG	133	TT = 0 TC = 14 CT = 7 CC = 0	21
C - 48	GATCTCGAGCCGAGATCTCGAGCCGA GATC TCGAGCCGAGATCTCGAGCCGAGATC TCGA GCCGAGATCTCGAGCCGAGATCTCGA GCCGAGATCTCGAGCCGAGATCTCGA GCCGAGATCTCGAGCCGAGATCTCGA GCCGAGATCTCGAGCCGA	156	TT = 0 TC = 24 CT = 12 CC = 12	48
C - 88	ATATATCTCTCTCTCTCCATATATCTC TCTCTCTCCATATATCTCTCTCTCTCCA TATATCTCTCTCTCTCCATATATCTCTC TCTCTCCATATATCTCTCTCTCTCCAT ATATCTCTCTCTCTCCATATATCTCTCT CTCTCC	144	TT = 0 TC = 40 CT = 40 CC = 8	88
T - 35	GCGATGTTATTATTATTATTAGCGATG TTATTATTATTATTAGCGATGTTATTA TTATTATTAGCGATGTTATTATTATTA TTAGCGATGTTATTATTATTATTAGCG ATGTTATTATTATTATTAGCGATGTTA TTATTATTATTA	147	TT = 35 TC = 0 CT = 0 CC = 0	35

Melanin Dissolution by UVA

A 0.5 mg/ml suspension of synthetic melanin (Sigma) in 50 mM phosphate buffer, pH 7.4 (or in water) was exposed to 200 kJ/m^2 of UVA. The same amount of melanin was incubated with 1 mM peroxynitrite stabilized in 7.8 mM NaOH (Millipore) for 54 min at room temperature (the time required to deliver 200 kJ/m^2 of UVA). Peroxynitrite actually dissolves the melanin in the first 5-10 seconds. Negative controls were untreated melanin and melanin treated with 7.8 mM NaOH (also incubated at room temperature for 54 min). Afterward, the melanin suspensions were centrifuged at 5000 x g for 10 min at room temperature and the supernatant transferred to new tubes leaving behind the pelleted melanin.

3D Distribution of Melanin Granules (melanosomes or melanin aggregates)

Z-stacks were captured on an LSM 710 DUO laser scanning confocal microscope using a 100X objective lens in DICIII mode, by directing the brightfield light path to the transmitted light photomultiplier tube (TPMT). This requires associating the brightfield lamp/TPMT track with a laser/PMT track which is then adjusted to 0% strength (we used the 543 nm HeNe laser). UVA- exposed and unexposed C57BL/6 melanocytes in PBS were used for imaging without incubation in formaldehyde, to avoid fixation artifacts. 3D projections were made using Volocity 3D Image Analysis Software (Perkin Elmer, Waltham, MA), using the Brightfield mode to invert dark to light in the TPMT Z-stacks (without inversion the extracellular space is an opaque block of white). Albino melanocytes (melan-c) that lack melanin-containing melanosomes showed no dark structures and were almost transparent.

Identification of Melanin Degradation Products by HPLC–MS

DHICA (0.1 mg/ml) was reacted with 5 μM horseradish peroxidase (Cat. P8375, Sigma) and 100 μM H_2O_2 (Cat. 2186-01, J.T. Baker) in 50 mM sodium phosphate buffer, pH 7.4 for 1 h at room temperature. Samples were injected into a Luna C18 reverse phase HPLC column (5 μm particle, 100 Å particle pore size, 250 x 4.6 mm, with C18 pre-column; Phenomenex, Torrance, CA). The liquid phase was 0.05% formic acid (v/v in water, solvent A) (Merck, Darmstadt) and acetonitrile (solvent B) (J. T. Baker). The gradient was a linear increase of solvent B from 1% to 10% in 25 min, after which the proportion was maintained at 10% of B for 5 min. Solvent B was then returned to 1% in 1 min and maintained at 1% for 5 min to stabilize the column. Detection was performed in a Micromass QuatroII mass spectrometer with Z-spray source (Waters, Milford, MA). The HPLC eluent was injected into the spectrometer at 150 ml/min. Ionization was electrospray in negative mode with conditions: source temperature, 100°C, desolvation temperature, 200°C; cone voltage, 20 V; dry gas, 300 L/h, nebulization gas, 15 L/h, mass range 100-600 *m/z*. Afterward the 224±1 *m/z* region was scanned for HPLC peaks.